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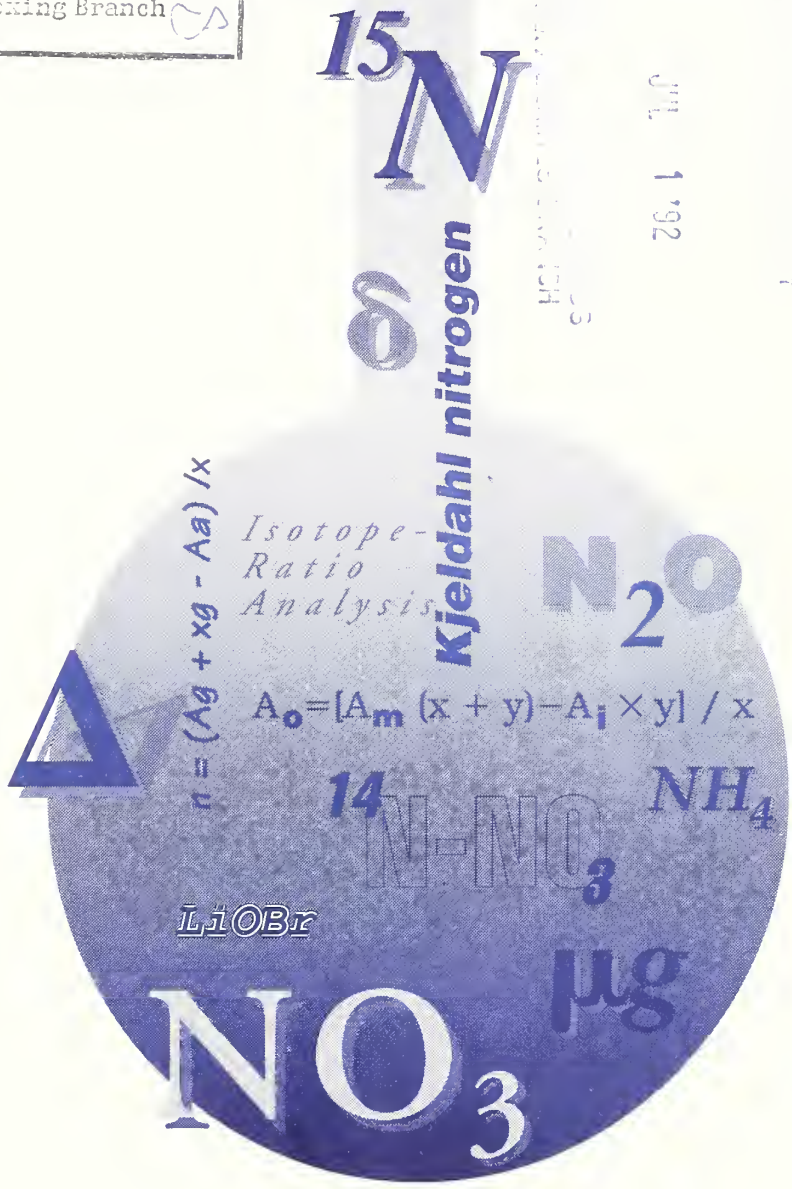

Abstract

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^{15}N technology is used to explore many agricultural research topics, including the movement of nitrates to groundwater, use of fertilizer nitrogen by plants, ways to increase nitrogen fixation, and effects of management practices on denitrification. This publication reviews ^{15}N procedures and methods for handling and collecting samples, introducing isotopes into plants and soils, and for performing Kjeldahl analyses, isotope dilutions, Rittenberg oxidation conversions for isotope-ratio analyses, and automated Dumas isotope-ratio analyses.

KEYWORDS: ammonium, automated analysis, denitrification, isotope-ratio analysis, Kjeldahl nitrogen, nitrate, nitrite, ^{15}N, ^{15}N analyses, nitrogen fixation, ^{15}N isotope, ^{15}N techniques.

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^{15}N Techniques and Analytical Procedures

Indo/U.S. Science and Technology Initiative

L. K. Porter and A. R. Mosier

Introduction

The stable N isotopes (^{14}N, ^{15}N) have been and are being utilized to study reactions and transformations of soil N, such as NH_4 fixation, chemo-denitrification, denitrification, mineralization-immobilization, and nitrification. These isotopes are also being used to study plant uptake and N recovery; the magnitude of symbiotic and nonsymbiotic dinitrogen fixation; N metabolism in plants, animals, and microorganisms; N losses and balances in natural environments and agroecosystems; NO_3 leaching; and the volatilization of N_2O into the atmosphere. Hauck and Bystrom (1970) published an extensive bibliography of ^{15}N applications concerned with various N research problems and methodology. Of the vast spectrum of ^{15}N applications, we have mentioned only a few.

Nitrogen isotope studies have revealed a great deal about many aspects of N in crop production and about the effects N compounds have on the air and water resources. The role of ^{15}N in unraveling nature's secrets appears to be ever expanding. Utilizing ^{15}N to explore the myriad of environmental, N-use-efficiency, and crop-production problems requires numerous methodologies, and the literature on these subjects is extensive (Bremner 1965, Johns 1971, Rennie and Paul 1971, Edwards 1973, Fiedler and Proksch 1975, Hauck and Bremner 1976, Bergersen 1980, Bremner and Hauck 1982, Buresh et al. 1982, Hauck 1982, Fiedler 1984).

It is hoped that this review of ^{15}N procedures will provide investigators, especially Indo/U.S. investigators, with some insights into many of the existing ^{15}N techniques available. Also it is hoped that some of the major potential sources of error will be delineated. However, this limited review cannot cover all the procedural problems or sources of errors that might be encountered. Each study is unique and requires its own approaches and procedures to accomplish its specific goals. The methodology finally selected for a given study may represent a compromise between various methodologies, and each investigator must strive to adopt an application, management, incubation, growth, or cropping system, and an analytical determination sequence that will provide the lowest level of error. Bremner et al. (1966) discussed assumptions and sources of errors in ^{15}N research. Martin and Ross (1968) have discussed the significance of errors in ^{15}N research. In crops and soils research, one of the major difficulties is caused by interactions between fertilizer N and soil N—the "priming effect" or "added nitrogen interaction" (ANI), which leads to confusion in interpretation of results. In a theoretical paper Jenkinson et al. (1985) showed how ANI's can cause real changes in N processes or can produce artifacts (pool substitution, isotopic exchange, or displacement). Legg and Meisinger (1982) discussed the errors that are accumulative and the statistical values that are important in making estimates of errors. The complex methodologies to be used in each study must be selected from the available literature and the experience, intuition, and judgment of the investigator. The objectives of each experiment will dictate the selection of experimental site, optimum plot size, number of treatments, replications, and sampling times (the experimental design needed to give credence to the study).

Isotope Dilution

The quantitative evaluation of pool sizes, metabolite pathways, chemical reactions, equilibrium products, or N-fractions at some sampling time in each system studied is based upon classical isotope dilution (Gest et al. 1947 and Inghram 1954). For example, when $^{15}NH_4$ fertilizer is mixed with soil, the $^{15}NH_4$ will equilibrate with the indigenous soil NH_4. This mixture will then take part in chemical and biological processes, and eventually the ^{15}N will be found in plant and soil biomass, organic matter, and inorganic N compounds. Once ^{15}N is detected in any tissues or compound we know qualitatively that the transformation has occurred, but quantitative estimates of the magnitude of the transformation are determined by dilution equations. Probably the main advantage of ^{15}N dilution is its usefulness to determine qualitative differences in treatments, i.e., types of cultivars, types of fertilizers, the timing and placement of fertilizer applications, and various forms of management, including irrigation, tillage, residue, herbicides, and pesticides. Using ^{15}N dilution calculations to make N balances for an entire cropping system or cropping sequence has many problems. These problems arise from errors associated with sampling (temporal and spatial variability), with measuring the soil bulk densities and soil weights for various soil profile depths, with measuring crop yields, and with determining the total N and the ^{15}N abundance of each component of the system.

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When planning a study, the researcher should make some rough estimates of the amount of unlabeled N in the various N pools that will be measured and thereby estimate the amount of dilution that will occur to his ^{15}N amendment. The ^{15}N enrichment of the added compound should be of sufficient magnitude that the ^{15}N can be easily detected after dilution. Many investigators (Gest et al. 1947, Jansson 1958, Huser 1966, Hauck and Bremner 1976, Fiedler 1984) have provided mathematical expressions that can be used to make dilution calculations. Hauck and Bremner (1976) discussed in detail numerous dilution equations, including the following:

$$A_2 = (T \times A_0 + P \times A_1)/(T + P)$$

where

T = total weight (g) of N in the labeled compound,
P = total weight (g) of N in the unlabeled pool,
A_0 = atom % ^{15}N of the labeled compound applied,
A_1 = atom % ^{15}N in the N pool before any ^{15}N addition, and
A_2 = atom % ^{15}N in the N pool after labeled addition.

The enrichment of the added compound should be of such magnitude that after dilution the percentage of ^{15}N in the new pool will be measurably different from natural N_2 abundance. The atom % ^{15}N of atmospheric N_2 is thought to be about 0.366 (Junk and Svec 1958), and this value for atmospheric N_2 is often used as a reference or standard for ^{15}N work. Atom % excess ^{15}N is often used in enrichment calculations and can be calculated as follows:

atom % excess ^{15}N = atom % ^{15}N in treated sample − atom % ^{15}N in untreated sample.

In naturally occurring soils, geological materials, and some biological systems, slight variations are found in the natural abundance of ^{15}N. These variations in natural ^{15}N abundance of natural systems may be caused by chemical and biological reactions that discriminate between ^{14}N and ^{15}N containing compounds, or they may result from analytical error in measurement (Wellman et al. 1968, Delwiche and Steyn 1970, Focht 1973, Blackmer and Bremner 1977). Although various discrimination reactions or mechanisms have been studied, we still can't account for some of the natural ^{15}N variations of N observed in soils. We also do not fully understand why natural ^{15}N values in soils are usually slightly higher than the atmospheric ^{15}N value (Cheng et al. 1964). Turner et al. (1983) suggested that the increased delta (δ) ^{15}N of agricultural soils is due, at least in part, to volatilization of depleted ^{15}N compounds during decomposition of N-rich plant material. Broadbent et al. (1980) studied the natural ^{15}N abundance in six soil cores taken from virgin and cultivated sites and concluded that the magnitude of natural ^{15}N variation at a given site, both laterally and vertically, is so great as to preclude tracing biological events by means of natural abundance. After studying ^{15}N in virgin soils and their fertilized analogues, Smith et al. (1983) concluded that it is impracticable to use variations in the natural abundance of ^{15}N for delineating soil nitrogen-cycling processes. Rennie et al. (1976) studied natural variations in soils and plants and stated "at this time, the use of fertilizer N enriched with ^{15}N offers major advantages over dependence on natural ^{15}N variations in studying complex soil biological and physical reactions associated with fertilizer N."

A literature survey of the natural ^{15}N abundance in soils shows a range of $\delta^{15}N$ values from about -8.4 to +16 (Cheng et al. 1964, Delwiche and Steyn 1970, Bremner and Tabatabai 1973, Rennie et al. 1976, Black and Waring 1977, Shearer et al. 1978, Broadbent et al. 1980, Karamanos and Rennie 1980). The values of $\delta^{15}N$ can be calculated from the following equation:

$\delta^{15}N$ = 1,000 (atom % ^{15}N sample − atom % ^{15}N std)/atom % ^{15}N std

If we assume that the atom % ^{15}N for the standard in this equation is identical to that for air (0.366), we can calculate that the atom % ^{15}N in numerous soils ranges from 0.3632 to 0.3729. Such slight variations in the natural ^{15}N abundance of soils usually has negligible effects on the interpretation of ^{15}N data, especially if the investigator treats the soil with a fertilizer that is sufficiently enriched with ^{15}N. If the atom % ^{15}N in untreated soil exceeds 0.3729, the investigator should look for sources of contamination in the analytical procedures.

When utilizing depleted ^{15}N fertilizers, it is necessary to maintain an untreated sample to determine the natural ^{15}N abundance of the system. Also it is probably wise to include check samples in studies involving the use of fertilizers enriched in ^{15}N.

Most magnetic focusing mass spectrometers (MS's) are capable of a precision of ±0.001 to ±0.003 atom % ^{15}N, and many of the more recent isotope ratio MS's are capable of measuring atom % ^{15}N with a precision of ±0.0005 or better. If one were using slightly enriched materials of approximately 0.36 atom % excess ^{15}N and could obtain a precision of ±0.001 atom % ^{15}N, the materials or fertilizer could be diluted approximately 360-fold. However, if an instrument capable of a precision of ±0.0005 atom % ^{15}N were used, the material could be diluted by 720-fold. As the limits of the instrument are approached, the ability to detect

slight enrichments diminishes. Therefore, it is important to know the capabilities of the MS's being used. Nevertheless, natural variations in soils and biological materials make 0.01 atom % ^{15}N excess a prudent level of detection that one might expect to attain.

The following example uses rough dilution calculations to demonstrate the atom % ^{15}N (in the soil and crop pools) that would result if an investigator applied fertilizers enriched with 0.7, 1.0, and 2.0 atom % ^{15}N. In this example, it is assumed that the surface soil is sampled to a depth of 0 to 15 cm and has a bulk density of 1.3 g/cm^3 and a total N content of 1 g N/kg soil. The rate of N applied is 100 kg N/ha, and it is assumed that half of this applied N will be assimilated by the corn *(Zea mays)* crop. Furthermore, it is assumed that the aboveground corn foliage and grain will contain 225 kg N/ha—50 kg N/ha from fertilizer and the rest of the N from indigenous sources in the 0-15 cm soil depth. The 0-15 cm depth is used in this example to simplify the calculations, although the crop (root geometry varies with species) assimilates soil and fertilizer N from soil depths of up to 120 cm and beyond. The total N pool prior to planting is determined as follows:

$$(1.3\ g/cm^3 \times 15\ cm \times 10^8\ cm^2/ha \times 0.001\ g\ N/g) = 1.95 \times 10^3\ kg\ N/ha$$

In the calculations below it is assumed that the natural ^{15}N abundance for the soil N is 0.366 atom % ^{15}N. If the fertilizer enriched with 0.7 atom % ^{15}N is added, the atom % ^{15}N in the total N pool of the soil is given by:

$$A_2 = (100 \times 0.7 + 1950 \times 0.366)/(100 + 1950) = 0.3823 \text{ atom \% } ^{15}N$$

If the fertilizers enriched with 1 and 2 atom % ^{15}N are added, the atom % ^{15}N values of the total soil N pool would be 0.3969 and 0.4457, respectively. A similar calculation can be made for the N pool of the crop (foliage and grain). For example, if fertilizer enriched with 0.7 atom % ^{15}N is added to the soil, the atom % ^{15}N in the crop N pool is given by:

$$A_2 = (50 \times 0.7 + 175 \times 0.366)/225 = 0.4402 \text{ atom \% } ^{15}N$$

If the fertilizers enriched with 1 and 2 atom % ^{15}N are added, the atom % ^{15}N values of the crop N pool would be 0.5069 and 0.7291, respectively.

When the crop is harvested, half of the applied N is contained in the crop. Therefore, for the treatment where 0.7 atom % ^{15}N was applied, the atom % ^{15}N in the soil N pool after harvest is given by:

$$A_2 = (50 \times 0.7 + 1775 \times 0.366)/(50 + 1775) = 0.3752 \text{ atom \% } ^{15}N.$$

If fertilizers enriched with 1 and 2 atom % ^{15}N are applied, the atom % ^{15}N values of the total soil N become 0.3833 and 0.4108, respectively. This example illustrates that after crop removal of fertilizer N and dilution of the 0.7 level of ^{15}N enrichment by the total N in the soil, the ^{15}N abundance is only 0.0092 atom % ^{15}N excess. Furthermore, this example shows that it is relatively easy to detect the isotope content in crop materials even at the lowest enrichment level. Also it is highly important to know the bulk density of the soil if the data are to be used to calculate ^{15}N balance data for fertilizer recovery.

Isotope Dilution in Dinitrogen Fixation

The most straightforward method for measuring N_2 fixation is to use $^{15}N_2$ in an enclosed vessel and directly measure the amount of label that is incorporated into microorganisms or plants from the enclosed ^{15}N. The gas can be handled, transferred, and easily recovered utilizing the high-pressure cylinder-cryosorption pump described by Porter (1970). This technique works successfully for small incubation flasks or short time exposures if the plant can be sealed in an airtight bag or gas lysimeter such as the one described by Ross et al. (1964, 1968).

However, the direct application of labeled N_2 to plants in the field is nearly impossible, and therefore in recent years microbiologists and legume breeders have been using ^{15}N isotope dilution to quantify N_2 fixation (LaRue and Patterson 1981, Chalk 1985, Phillips et al. 1986, Rennie 1986, Vose and Victoria 1986). The assumptions, advantages, and disadvantages of this technique in measuring N_2 has been discussed by these investigators. The isotope dilution used in quantifying N_2 fixation is not the same as the classical isotope dilution outlined by Gest et al. (1947) or Hauck and Bremner (1976). In the technique for quantifying N_2 fixation, the legume assimilates labeled fertilizer N that has been diluted by soil N. The labeled N is further diluted in the plant as the nodule fixes natural abundance $^{15}N_2$ from the atmosphere. The method was introduced by McAuliffe et al. (1958) and expanded in studies by Fried and Broeshart (1975) and Fried and Middelboe (1977). To determine the atom % excess ^{15}N taken up from the soil, the investigator must use for comparison a reference plant that can assimilate soil N and tagged fertilizer N but is resistant to *Rhizobium* infection and therefore unable to fix N_2. Various grasses and nonnodulating legumes have been used as

reference plants. An assumption is that the reference plant will absorb the same proportion of soil N and tagged fertilizer N throughout the growing season as the nodulated legume. However, assimilation by the nonnodulated legume may not be the same as the nodulated legume, and this is one of the limitations of the method. For example, a reference plant that is resistant to *Rhizobium* infection is probably resistant to infection by vesicular-arbuscular-mycorrhiza (VAM), and it has been shown by Ames et al. (1984) that VAM can alter the uptake of N derived from indigenous soil sources. Legume plants assimilate N from the soil profile and throughout the cropping season. Therefore this method of using nodulated and nonnodulated legumes permits investigators to determine dinitrogen fixation values that have been integrated over time and space. It is important to apply small amounts of ^{15}N (2 kg of fertilizer N per ha containing 25 atom % ^{15}N or greater enrichments) so that inorganic N does not depress N_2 fixation or inhibit normal root nodule development. Legg and Sloger (1975) proposed a method in which the inorganic tagged N is converted into an organic form through the addition of sucrose prior to initiation of the N_2 fixation study in order to avoid the depressing effect of mineral N. However, the readily available sucrose C alters microbial activities and interferes in the normal immobilization-mineralization cycle of the soil. The magnitude of error resulting from such altered microbial activities needs further study.

The following equations are used to calculate N_2 fixation from isotope dilution:

$$\% N_2 \text{ fixed} = 100\,(1 - \text{atom \% excess } ^{15}N \text{ in the fixing crop})/\text{atom \% excess } ^{15}N \text{ in the nonfixing crop}$$

$$\text{Amount } N_2 \text{ fixed} = (1 - \text{atom \% excess } ^{15}N \text{ in the fixing crop} \times \text{total N})/\text{atom \% } ^{15}N \text{ in nonfixing crop}$$

Methods for Applying ^{15}N Isotopes into Soils and Plants

The application of labeled N fertilizers requires very careful measurement of the amounts of N applied. Any source of enriched ^{15}N (gas, salt, or organic substance) should be analyzed for its ^{15}N content before initiating a study. When making dilutions to obtain a compound with a specific atom % ^{15}N value for an experiment, it is important that the compound be homogeneous. The best way to obtain homogeneity is to dissolve the salts or compound before applying. If it is necessary to apply the diluted compound dry, it should be dissolved and redried before it is applied. There are many ways to introduce isotopes into the system to be studied. The objectives and available equipment will determine how the isotope is applied. Probably the simplest and most straightforward methods are those involved in metabolic studies. In animals the isotope-enriched substrate or compound can be injected or fed directly; in plants the labeled substance can be mixed in a nutrient solution for direct plant assimilation. An equilibrium is obtained where uniform mixing occurs, and the enriched compound undergoes a predictable dilution from nonenriched compounds. However, problems can occur even in the most straightforward applications. For example, when ryegrass and wheat were exposed to >99 atom % ^{15}N-NO_3, previously absorbed ^{14}N-NO_3 appeared in the nutrient solution, i.e., with the influx of tagged NO_3 into the plant root there was an efflux of previously absorbed nontagged nitrate (Jackson 1978).

Foliar application of fertilizer N has been of interest to agronomists for many years, and a number of N carriers have been tried. In recent years it was thought that foliar-applied urea might be an effective way of providing supplementary nitrogen to corn as it approached maturity. This idea probably deserves more study with isotopes. Volk and McAuliffe (1954) studied urea absorption by tobacco leaves and found that many factors may affect absorption. They suggested that internal factors in the leaf undergo diurnal fluctuations and play an important role in urea absorption. They found urea absorption over a 4-hour period was 3 to 10 times greater during the night than during the day. Absorption was also 3 times greater in the morning than in the afternoon. The absorption of urea from buffered solutions was related to both pH and the buffer composition. Also urease activity has been observed on plant leaves, so one might expect that pH, salts, and organic substrates would affect the hydrolysis of urea and the subsequent volatilization of ammonia from the leaf surface. Wetting agents have been used to help coat the leaf surface with a tagged compound, and tagged compounds can be directly injected into some monocots. Another method of introducing the tagged compound into a plant is to thread a needle with cotton thread, run the needle through the stem of the plant and back, and put the ends of the cotton thread into the tagged solution. As the plant transpires, the tagged compound is wicked into the plant (Comar 1955, Porter 1957). The technique works well for dicots but damages intercalary meristems that are found at the internodes of many monocots.

Probably the major uses of ^{15}N compounds have been in studying plant uptake, fertilizer N-use efficiency, ^{15}N balance in cropping systems and natural ecosystems, losses of fertilizer N applied to soils, and the turnover of N in various soil-plant N pools. The application of fertilizer N compounds to soils is fraught

with problems, especially when applying fertilizer N in the field. In greenhouse studies the fertilizer usually is mixed thoroughly with the soil in some sort of blender (one of the most common is the Twin Shell Dry Blender manufactured by Patterson-Kelly Co., a Div. of Taylor-Harsco Corp., East Stroudsburg, PA). In the field one must choose between using microcylinders (10-30 cm diameter), lysimeters, or macroplots that vary in size from a few m^2 or larger. The cost of the isotope largely limits the size of the larger plots. Microcylinders reduce the amount of ^{15}N fertilizer required and decrease the amount of plant tops, roots, and soil that must be sampled and processed. The cylinders are usually steel or plastic pipe cut to the desired length. If other materials are used to construct cylinders, only materials that are nontoxic to plants and microbes should be used. For example, galvanized sheeting may be toxic to microbes. The cylinders must be sufficiently long to prevent outside roots from taking up nutrients inside the cylinder. Carter et al. (1967) found that a 60-cm-long cylinder was sufficient to prevent feeding by external Sudan grass roots. As the diameter and length of the cylinder increase, it is more difficult to drive it into undisturbed soil. The major disadvantage of the microcylinders is in obtaining a reliable estimate of crop yields and N content and extrapolating these values to a field basis. More reliable estimates can be obtained by fertilizing an area outside the cylinder with nontagged N and then harvesting this larger area to estimate crop yield and N content. Trevitt et al. (1988) studied the use of microplots in assessing the fate of urea in flooded rice. The results showed that the use of enclosures can retard urea hydrolysis, suppress the maximum daytime pH values, and reduce the potential for ammonia volatilization. Their analysis suggests that errors due to shading from the walls are acceptable when 90% or more of the incident solar radiation always penetrates to the enclosed floodwater. If this is the case, then square plots with opaque walls must be at least 1.2 m long and cylindrical plots must be at least 1.2 m in diameter when the wall height is 0.1 m above the floodwater.

Labeled fertilizers may be mixed with, broadcast on, sprayed on, and injected into soil or mixed with irrigation water, but volatile losses of NH_3 from such applications can cause significant balance-sheet errors. When applying NH_4 salts and urea on a soil surface, it is important to immediately till the soil several inches deep so that the fertilizer is mixed into the soil (rototilling, plowing, etc.) to help avoid losses of NH_3 by volatization. Immediate tilling is especially needed if $(NH_4)_2SO_4$ is applied to calcareous soils (Fenn and Kissel 1973). The extent of the losses depends upon the pH and buffer capacity of the system (Avnimelech and Laher 1977). Volatile losses of NH_3 can also occur after urea is applied to the floodwaters of rice (Macrae and Ancajas 1970, Ventura and Yoshida 1977, Mikkelsen et al. 1978, Vlek and Stumpe 1978, Vlek and Craswell 1979, Vlek et al. 1980, Freney et al. 1981). This volatilization of NH_3 can be reduced by placing the urea deeply in the soil below the floodwater (Craswell and Velk 1979, Cao et al. 1984). Volatilization of NH_3 from floodwater is directly related to the concentration of NH_3 in the water (Mikkelsen et al. 1978, Vlek and Stumpe 1979, Vlek and Craswell 1981). The NH_3 concentration in the water is affected by the NH_4 concentration, pH, and the CO_3 or buffer capacity of the floodwaters. The CO_3 concentration is strongly influenced by algal photosynthesis, which causes diurnal fluctuations in the pH (Fillery et al. 1984). Urea hydrolysis produces NH_3 and $(NH_4)_2CO_3$. The $(NH_4)_2CO_3$ is highly unstable in water and influences the buffering capacity of the waters (Stumpe 1981).

A uniform application is difficult to achieve when applying fertilizer salts by hand. Removing the soil and mixing the fertilizer with the soil is probably the only way to achieve any high degree of uniformity. Uniform mixing of tagged fertilizer with soil is possible when plot size is limited to small cylinders, but labor associated with mixing becomes exceedingly costly as plot size increases. The use of macrofield plots makes it possible to apply fertilizer and irrigate more conventionally but decreases the uniformity of fertilizer application. Woodcock et al. (1982) designed and built an apparatus for applying ^{15}N-labeled fertilizer uniformly to small field plots. Their apparatus applies aqueous solution evenly in rows 5 cm apart over an area measuring 2m by 1m. This apparatus is light enough to be carried by hand and can apply fertilizer to 20-40 plots in a working day. The fertilizer is surface applied without gaps or overlaps whether it is operated slowly, rapidly, or even stopped during a run. A disadvantage of this apparatus is that the plot size must be in multiples of 2m by 1m. Such a restriction on plot size is fine for wheat or a grass crop where fertilizers are often surface applied. However, for row crops, such as sugar beets, potatoes, sorghum, and corn, fertilizer N is usually applied by knifing a band of fertilizer into the soil, and the spacing of the fertilizer band is usually dictated by the farm machinery available. Broadbent and Carlton (1980) developed an applicator that meters out precise volumes of fertilizer solution and injects the solution into the soil through two shanks, one on either side of the row. The tagged solution is pumped through a positive displacement pump linked to the wheel drive so that a known volume is delivered per unit area regardless of the speed traveled. It appears that this device is most suitable for macroplots, where depleted ^{15}N fertilizer would be banded near the seed and tagged fertilizer would be applied in a split application. Sanchez and

Blackmer (1988) have developed an apparatus for the band application of ^{15}N-labeled anhydrous NH_3 to small plots. The main deficiency of this apparatus is that the capillary tube is drawn through the soil by a hand-driven winch, probably giving a nonuniform application. It appears that a uniform application could be achieved if the winch was driven by an electric motor geared to give the desired rate of movement. The motor could be battery powered in order to make the apparatus portable. Surface applications of fertilizers are possible by spraying or applying the dry fertilizer with a spreader. However, such applications should probably be made in more than one direction over the plot.

Banding of fertilizers causes extreme soil heterogeneity and raises the question of how to take soil samples or gas samples to avoid spatial variability. Carter et al. (1967) found wide variations in recovery of ^{15}N among core samples taken from microcylinders where fertilizer was applied by banding and concluded that it is necessary to remove, weigh, mix, and subsample the entire soil mass from within the cylinders. The soil is usually removed by soil depth or layers and then the subsample mixed individually. However, removing and mixing soil from macroplots or from rows where the fertilizer has been banded is nearly impossible to do with any precision. Large sampling errors are associated with taking soil cores from large macroplots. An appreciation that spatial variability imposes extreme limits on calculating ^{15}N balances and extrapolating soil core data to large land areas is needed. Biggar (1978) and Nielsen et. al. (1982) pointed out that large uncertainties exist in balance-sheet studies even for small field plots. The technology for sampling and assessing spatial and temporal variability of macroplot or field plots under a given cultural practice remains largely underdeveloped. Spatial and temporal variability also limits our ability to measure volatile nitrogenous gases or to sample soil solutions by suction-cup devices.

If ^{15}N is used to measure the magnitude of N_2 fixation, the investigator should consider the spatial variability of residual NO_3 at various depths in the soil profile. The control plants and fixing plants for a N_2 fixing experiment should exploit the same soil volume.

Sample Collection and Handling

The yield or weight of the plant material multiplied by its N content, the soil's bulk density multiplied by its N content, and unit area are used directly in the calculation of ^{15}N balances. In field studies, usually only small areas are harvested, and the data are extrapolated to give yields per hectare or per acre. Such extrapolations may introduce large errors in N balance studies. Generally the greatest sources of error in ^{15}N research are not associated with chemical or isotope determinations but are associated with poor estimates of yield, soil bulk density, and sample variability associated with spatial heterogeneity. Other sources of error occur in subsampling or result from lack of homogeneity in sample preparation. For greenhouse pots and small microcylinders, the total plant or soil can be harvested, dried, weighed, and ground prior to subsampling for chemical determinations. However, for field macroplots it is nearly impossible to harvest the entire plot and process it. Usually the macroplot fertilized with tagged N is surrounded by a plot fertilized with untagged N. Plants in the outside plot can be machine harvested to estimate the yield. When the macroplot is sampled for ^{15}N, border effects may be a problem. Lateral feeding from roots of outside plants can lower the ^{15}N abundance of border plants (Bartholomew 1964). Sanchez et al. (1987) modeled the lateral movement of tagged fertilizer N by sampling corn grain at various positions inside and outside ^{15}N plots. Using a corn crop, they found that unconfined plots (2m by 2m) are sufficiently large for determining recovery of fertilizer N under most conditions. Follett et al. (1990) studied border effects for wheat microplots and observed that once inside or outside of the border of the ^{15}N-fertilized microplot at a distance of 0.46 m there is no difference in enrichment or in total ^{15}N uptake into plant tops. Olsen (1980) indicated that accurate values for corn uptake of N could be obtained by sampling the center plants of a 3-row plot at least 214 cm long and 71 cm apart. Usually border rows are not harvested except to explore lateral feeding of plant roots or lateral movement of nitrates. Only rows more central in the plot are harvested. The length of row to be harvested depends on the size of the plot, the number of people available to process the harvested materials, and number of ovens available to dry the materials. Depending on the maturity of the crop, whole-plant samples may be ground or the plant may be separated into components, such as grain, chaff, straw, leaves, etc., and then ground. As the plant nears maturity it is more difficult to obtain a homogeneous sample if the entire plant is ground. Dividing the plant into components provides more homogeneous samples but multiplies the number of samples to be analyzed.

When trying to determine inorganic N (NO_3 or NH_4) in soil samples, if possible soil samples should be moist when processed; subsamples should be taken to determine moisture content and immediately extracted and analyzed. However, field sampling usually involves some delays in transporting and processing of samples before analysis. Taking large numbers of soil samples or soil cores further increases the possibility of delays. During delays rapid changes in the inorganic forms of N in the samples may occur due to biological reactions (Keeney and Nelson 1982). The most commonly used method for preserving soil samples appears to be freezing or rapid drying. As Keeney and Nelson have pointed out, results of studies to evaluate the effects of sample pretreatment on inorganic N forms have been mixed. Freezer storage is generally recommended (Nelson and Bremner 1972). However, finding freezer space for numerous samples may be a problem. Quickly air drying the sample on a greenhouse bench and storing the sample in a sealed container may be the best alternative when numerous samples must be processed. Soil samples from a puddled rice crop require large volumes of water to be evaporated, and volatile NH_3 may be lost in the evaporation process.

Extreme care should be exercised during the initial grinding and subsampling of soil and plant samples. If possible, the entire sample should be coarsely ground and the mill thoroughly cleaned between samples to avoid cross-contamination. To help avoid cross-contamination of samples during grinding, distilling, or isotope analysis, it is best to start with the lowest ^{15}N abundance samples, followed by samples expected to have progressively higher ^{15}N abundance. From each sample, a portion is stored in a tightly sealed bottle to avoid any NH_3 adsorption from the atmosphere. Small amounts of these coarsely ground materials should be further ground to at least 0.075 mm (200 mesh) size to obtain a homogeneous sample. This grinding can be accomplished by ball milling or using a wiggle bug apparatus (Pica Blender from Cianflone Scientific Instruments Corp., 4898 Campbells Run Road, Pittsburg, PA, or Impact Grinder and Blender from Spex Industries, Inc., 3880 Park Ave., Edison, NJ). Centrifugal mills, such as the Retsch from Brinkman Industries, can also be used to rapidly grind samples to produce the desired 0.075 mm homogeneous sample for Kjeldahl nitrogen or automated Dumas analyses. The finely ground sample should be dried at 65 °C and stored in a desiccator prior to analysis.

Kjeldahl Analysis

Digestion

Kjeldahl digestion is the classical procedure for converting organic N in plants and soils to NH_4 salt. Both macro and semimicro Kjeldahl procedures are used (Bremner 1960, 1965). The sources of error in Kjeldahl procedures have been discussed by many authors (Hauck and Bremner 1964, Bremner 1965, Bremner et al. 1966, Martin and Ross 1968, Fiedler and Proksch 1975, Bremner and Mulvaney 1982). The NH_4 is recovered and oxidized by alkaline hypobromite converting the NH_4 to N_2 for ^{15}N analysis.

Transferring a weighed sample to the bottom of a digestion flask or digestion tube is a potential source of error. Static electricity attracts both plant and soil materials to glass walls of the digestion tube and long-stem funnels. Trying to wash adhering materials from long-stem funnels or the glass wall of the neck of a digestion flask is fraught with problems. We have attempted to overcome this problem by constructing slender stainless steel spoons that fit through the narrow neck of semimicro digestion tubes or flasks. The sample can be weighed directly into such a spoon. A hemostatic forcep can be attached to the short spoon handle so that the spoon can be guided directly to the bottom of the digestion flask or tube.

Charring of plant samples during digestion may occur and is a problem that can be partly overcome by allowing the sulfuric acid to predigest the samples at room temperature overnight. The samples must be protected from atmospheric contamination during this predigestion period. Careful control of the digestion temperature between 360-370 °C is required to assure complete sample digestion without incurring any loss of N (Lake et al. 1951, McKenzie and Wallace 1954, and Bremner 1960). The temperature attained is controlled by the ratio of the digestion salt-catalyst to acid (Bremner 1960). A ratio of 0.66 g K_2SO_4/ml of acid is needed to attain 370 °C. The potential problem of sample overheating can be eliminated by using a digestion block that has a built-in thermocouple and temperature controller that can maintain 370 °C. Compared to digestion heating racks, aluminum digester blocks provide better temperature control and can be used to process samples (up to 40 samples at one time) with less attention. Calibrated digestion tubes are used with the blocks and require less space than digestion flasks. The digests can be brought to volume in the calibrated tubes, and small aliquots can be taken for the colorimetric determination of NH_4 (Schuman et al. 1973). Bremner and Mulvaney (1982) recommended a Kjeldahl digestion period for soil samples of 5 hours to ensure complete conversion of all

forms of organic N to NH_4. However, Bremner (1960) showed that the conversion in soil materials can be complete in as little as 1 hour when salt/acid ratios vary between 0.66 and 1.33. Ratios of 1.66 or greater caused losses of N even when the refluxing continued for only 1 hour. During digestion sulfuric acid is consumed in the oxidation of carbon. This consumption of acid can readily change the acid/salt ratio. Fiedler (1984) presented tables of factors for many compounds and materials that permit rapid calculation of the acid consumed per gram of compound or material being digested.

Nitrate and NO_2 in plant tissues and soil samples can be a source of error in Kjeldahl determinations. These inorganic forms of N must be reduced to NH_4 before the digestion takes place in order to obtain a total N value. Without such pretreatment the organic C oxidation will reduce part of the NO_3 in the plant tissues, resulting in error (Pace et al. 1982); thus the assumption cannot be made that Kjeldahl digestions will cause the loss of all the NO_3 in the plant tissue without the prereduction treatment. Often the NO_3 and NO_2 sources of N in plant and soil samples can be highly labeled, and loss or lack of recovery may cause serious error in ^{15}N balance studies. The need to reduce NO_3 in Kjeldahl analyses was discussed by Bremner and Mulvaney (1982). The reduction may be accomplished by first complexing the NO_3 with salicylic acid and reducing the complex with $Na_2S_2O_3 \cdot 5H_2O$ (Bremner 1965). As discussed by Bremner and Mulvaney (1982) and Hauck (1982), various investigators have questioned the reliability of the salicylic acid procedure to totally recover NO_3 in soil and plant materials. It has been suggested that traces of water in the sample materials may interfere with the formation of the salicylic-NO_3 complex, although Bremner (1965) suggested that no such interference occurs. Liao (1981) used Devarda's alloy as the reducing agent in the pretreatment of the sample prior to digestion. This method is quite sensitive to the sulfuric acid concentration (optimum concentration 6 N). The method devised by Liao hasn't received widespread acceptance. Bremner and Mulvaney (1982) suggested that the preferred method for reducing NO_3 in total N analysis in ^{15}N research is the permanganate reduced-Fe modification introduced by Bremner and Shaw (1958). In this method the sample is pretreated with $KMnO_4$ and H_2SO_4 to oxidize the NO_2 to NO_3 and then the NO_3 is reduced by ferrous ions to NH_4 in the acid solution. The following two methods for the reduction of NO_2 and NO_3 in sample materials are from Bremner and Mulvaney (1982), and the procedures for the methods largely come from Bremner (1960, 1965) and Bremner and Mulvaney (1982):

Permanganate Reduced-Iron Modification for NO_3 and NO_2

Reagents

1. Potassium sulfate-catalyst mixture: Weigh out 200 g of K_2SO_4, 20 g of cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), and 2 g of Se. Handle the Se in a hood carefully so as not to breath the Se dust or allow Se to come in contact with the skin. Powder the reagents separately before mixing. Mix the ingredients, and then grind the mixture in mortar to powder the cake that forms during the mixing.
2. Concentrated H_2SO_4.
3. Potassium permanganate ($KMnO_4$) solution: Dissolve 25 g of $KMnO_4$ in 500 ml of water, and store the solution in an amber bottle.
4. Dilute H_2SO_4: To 500 ml of water in a 2-liter Pyrex flask, add slowly and with continuous stirring, 500 ml of concentrated H_2SO_4.
5. Reduced Fe: Use a finely divided product and sieve to remove any material that does not pass through a 100-mesh screen.
6. n-octyl alcohol.

Procedure

Place a sample that preferably contains 2 mg N or more in a microKjeldahl digestion flask or tube, add 1 ml of $KMnO_4$ solution, and swirl the flask for about 30 sec. Hold the container at a 45-degree angle, and slowly pipette 2 ml of dilute H_2SO_4 down the inside of the container while swirling the container continuously. Allow the ingredients to stand for 5 min, and add 1 drop of the n-octyl alcohol. Add 0.5 g of reduced Fe through a long-stem funnel, and allow the solution to stand until the initially strong effervescence has ceased. Then gently heat the solution on the digestion block or heating element for 45 min (the heating should not lead to a significant loss of water). Allow the mixture to cool, add 2.2 g of K_2SO_4-catalyst mixture and 3 ml of concentrated H_2SO_4, and then heat the container cautiously until the water is removed and frothing ceases. Increase the temperature of the solution to 370 ^{o}C, and allow the sample to digest for 3 hours after the solution clears. Allow the digest to cool slowly, and then cautiously add 15 ml of distilled water. Swirl until the digest is completely mixed or, if digest is caked, until it disintegrates and dissolves. Save the digest for the distillation procedure that is described later in this section.

Salicylic Acid-Thiosulfate Modification for NO_3 and NO_2

Reagents

1. K_2SO_4-catalyst mixture: Described in the permanganate reduced-Fe method.
2. Salicylic acid-sulfuric acid mixture: Dissolve 25 g of

salicylic acid in 1 liter of conc. H_2SO_4.
3. Sodium thiosulfate pentahydrate ($N_2S_2O_3 \cdot 5H_2O$): Powder crystals of the sodium thiosulfate pentahydrate to pass a 60-mesh screen.

Procedure

In a Kjeldahl calibrated digestion tube, place a sample that preferably contains at least 1 mg N (1 mg N can usually be obtained with 0.1 g of plant tissue, but for soil samples 1 g or more may be required). Using 1 g or more of soil in the semimicro container usually causes bumping, which may be lessened by adding glass beads or pumice. Add the salicylic acid-sulfuric acid mixture to the sample: 3 ml for a plant sample and 6 ml for a soil sample. Gently swirl the container until all materials are wet. Allow the mixture to stand for several hours or preferably overnight if it is protected (flasks or tubes are stoppered) from the atmosphere. Add 0.5 g of sodium thiosulfate pentahydrate to the mixture through a dry long-stem funnel. Cautiously and slowly heat the mixture until the frothing has ceased and a temperature of 200 °C is attained (this heating usually takes about 1 hr). Allow the mixture to cool, and add the K_2SO_4-catalyst mixture through a long-stem funnel while maintaining a ratio of 0.66 g K_2SO_4/ml concentrated H_2SO_4 in the mixture. Slowly heat the mixture to 370 °C. After the mixture clears, reflux it for at least 2 hours if it contains plant materials and reflux it for 3 hours if it contains soil materials. After the digestion is finished, allow the digest to cool, and then add distilled-deionized water slowly while stirring until the liquid is about 2 cm below the graduation mark. Allow the mixture to cool to room temperature before bringing it to final volume. Save this digestion mixture for the NH_4 determination and NH_4 recovery procedures described below.

Procedure for Determining and Recovering NH_4 from Kjeldahl Digests

If digestion-distillation flasks are used, the normal mode of measuring the NH_4 in the digest is as follows: make the digest alkaline, steam distill the digest, and then titrate the distillate. This mode of operation can result in cross-contamination problems (Hauck and Bremner 1964, Bremner 1965, Bremner et al. 1966, Newman 1966, Martin and Ross 1968, Saffigna and Waring 1977, Reeder et al. 1980, Buresh et al. 1982, Mulvaney 1986, Vanden Heuvel and Giamalva 1988). Cross-contamination error can be serious if one is trying to utilize variations in natural ^{15}N abundance as a tracing aid or trying to measure natural ^{15}N discrimination reactions. The error is caused by adsorbed NH_4 ions on the walls of stills, condensers, beakers, electrodes, O-rings, plastic, and rubber tubing. If possible eliminate plastic and rubber parts from distillation apparatus because adsorbed NH_4 is not easily removed from these items. Remove adsorbed NH_4 in glassware such as beakers, flasks, and digestion tubes by rinsing them with 2% HF, 2% acetic acid, or EtOH/KOH (Rennie and Paul 1971, Reeder et al. 1980, Hauck 1982, Mulvaney 1986, Porter et al. 1986). The still can be a major source of cross-contamination unless precautions are taken. It is time consuming to disassemble a distillation still between each sample. The still can be constructed of stainless steel to minimize cross-contamination (Buresh et al. 1982). Steaming the stainless steel system for 2 min between samples largely eliminates adsorbed NH_4 (Saffigna and Waring 1977, Buresh et al. 1982, Vanden Heuvel and Giamalva 1988).

If an all-glass still is used, the following procedures will reduce cross-contamination error. Steam distill two aliquots of each sample whenever possible—use the first distillate for titration, and retain the second for isotope-ratio analysis (Mulvaney 1986). Distill sufficient NH_4 to develop the desired N_2 inlet pressures for MS analysis. Most of the older isotope ratio MS's require about 1 mg of N, but this depends upon the volume of the expandable bellows of the MS inlet. When it is not possible to distill two aliquots of a sample, use the following procedure between samples: Steam distill 1 ml of 1 M formic acid; then change the solution in the distilling flask to 25 ml of 95% ethanol, and steam distill the ethanol 3 min. When preparing samples (mixing, grinding, weighing for digestion, distilling, titrating, and converting samples to N_2 gas), start with the samples known to contain the lowest ^{15}N abundance and work to the samples suspected or known to contain the highest ^{15}N abundance. The amount of adsorbed NH_4 in a distillation still is constant (usually between 1 μg and 5 μg, depending upon the design, i.e., the surface area of the still). Since the amount of adsorbed NH_4 in the still is constant, as the concentration of NH_4 in the sample increases, the dilution error decreases.

If digestion tubes are used, NH_4 content of the digest can be determined by either of the following procedures: Transfer the digest into a distillation flask, make alkaline, steam distill, and follow by either a potentiometric or colorimetric titration (Crooke and Simpson 1971, Keeney and Nelson 1982); or bring the digestion tube to volume, and colorimetrically determine the NH_4 content of a small aliquot of the digest (Schuman et al. 1973). The use of a small aliquot preserves most of the digest for NH_4 recovery by either alkaline distillation or alkaline diffusion (O'Deen and Porter 1979, Adamsen and Reeder 1983). In the diffusion process, make the remaining digest alkaline with NaOH, and place a small vial of HCl (3 ml of 1 M HCl) in the top of the screw-capped diffusion tube. The

design of the screw-capped diffusion tube is given by O'Deen and Porter (1979). Seal the tube and heat to 130-140 °C on a digestion block positioned in a fume hood. The NH_3 released is trapped by the acid. The long diffusion tube functions as an air condenser, helping to keep the acid vial from accumulating water. Also, the hot and cold portions of the tube cause a convection movement in the solution that continually stirs the digest. The diffusion period varies from 18-48 hr depending upon the temperature and volume of the digest in the tubes (O'Deen and Porter 1979, 1987). The digest can also be transferred to urine specimen cups and NH_3 diffused from an alkaline media (MacKown et al. 1987). When using plastic containers, the diffusion process takes 6 to 13 days. In NH_3 recovery either by diffusion or distillation it is important to be sure complete recovery occurs in order to avoid isotope fractionation (O'Deen and Porter 1979, 1980). The diffusion technique is slow compared with steam distillation but is less labor intensive and less subject to cross-contamination.

Some soils may contain appreciable amounts of fixed NH_4. The normal Kjeldahl digestion procedure cannot be used to measure the fixed NH_4-N in some soils (mostly heavy clays) unless the soil is pretreated with HF (Stewart and Porter 1963). Procedures for obtaining fixed NH_4-N by HF pretreatment are outlined by Bremner and Mulvaney (1982).

Reagents used in Kjeldahl analyses contain small amounts of N (Kjeldahl blanks). The N in the reagents causes a slight dilution of the sample ^{15}N and thereby alters ^{15}N abundance of the sample. It is extremely important to account for the dilution caused by the blank N to avoid underestimating N recovery or the ^{15}N balance of a system.

Procedure for Diffusing NH_3 from Kjeldahl Digests

Construct screw-cap digestion diffusion tubes, 2.5 by 30 cm, by cutting off the bottoms of screw-cap culture tubes (Corning #9826) and glass blowing them to calibrated Folin-Wu digestion tubes (Corning #7940). See O'Deen and Porter (1979) for an illustration of the diffusion tubes. Caps with conical polyethylene liners (Poly-Seal Caps) provide excellent seals. Make small glass indents 7.5 cm from the top of the tubes to support the NH_3 absorption traps, which are 1.5 by 5 cm shell vials containing 3 ml of 1 N HCl.

Make the digests alkaline with 10 M NaOH, and carefully place the NH_3 traps in the tops of the diffusion tubes. Cap the tubes, and place them in a digestion block that is in a safety hood. Heat to 140 °C, and maintain for 48 hours. At this temperature the diffusion tubes are under considerable pressure and may be a hazard to the operator. Alkali causes erosion of the glass tubes, so the hazard increases with length of use. Usually the thinning of the diffusion tube's glass walls occurs at the bottom of the tube, which is down in the heating block; thus the hazard is decreased somewhat, but safety precautions should always be observed when the glass diffusion tubes are at elevated temperatures. After diffusion, allow the tubes to cool, and carefully remove the NH_3 absorption traps. Evaporate the excess HCl in the vials with NH_3-free air at 95 °C in a fume hood. Use parafilm to cap the vials, and store them for isotopic analysis of the dry NH_4Cl salt.

Care must be exercised in the evaporation of the samples to avoid losses of NH_4 (Reeder 1984). Do not dry the samples at temperatures above 95 °C, and do not leave dry samples on the heating block after drying. Cap the shell vials with Parafilm and store for isotopic analysis. Hauck (1982) recommends trapping NH_3 in H_2SO_4 because the $(NH_4)_2SO_4$ formed is stable up to 235 °C. However, it is difficult to remove the excess H_2SO_4. Alkaline hypobromite is used to convert the recovered NH_4 to N_2 for isotopic analysis, and excess H_2SO_4 causes decomposition of the alkaline hypobromite with release of Br_2. The excess sulfuric acid can be converted to salt by adding K_2SO_4 to the concentrated acid, resulting in the formation of KNH_4SO_4 or $KHSO_4$ (Brooks 1985). A special gas-scrubber-forced air system can be used to speed evaporation and minimize atmospheric NH_3 contamination (Lober et al. 1987).

Distillation of NH_3 from Kjeldahl Digests

Reagents

1. Sodium hydroxide (approximately 10 M): Place 3.2 kg of reagent grade NaOH in a heavy-walled 10-liter Pyrex carboy marked to indicate a volume of 8 liters. Add 4 liters of CO_2-free distilled water to dissolve the alkali. Put a rubber stopper in the neck of the carboy, and allow the solution to cool. Add CO_2-free distilled water until the solution volume in the carboy is 8 liters, and mix the contents. Protect the reagent from atmospheric CO_2.
2. Boric acid solution: Place 80 g of boric acid in a flask marked to indicate a volume of 4 liters. Add 3,800 ml of water. Place the flask on a stirrer-heater; heat and stir the solution until the boric acid is dissolved. If the distillates are to be titrated potentiometrically, add 75 g KCl, and adjust the boric acid to a pH of 5.1 by adding 0.1 M NaOH; bring the solution to a final volume of 4 liters.
3. Boric acid indicator solution: Dissolve 0.099 g of bromocresol green and 0.066 g of methyl red in a small amount of ethanol. Add 80 ml of this indicator to the boric acid solution (prepared as described above), and

then slowly add 0.1 M NaOH to the boric acid solution until it turns reddish purple. Add distilled water to bring the boric acid solution to 4 liters, and thoroughly mix the solution. Use this boric acid indicator solution if distillates are to be visually titrated.
4. Prepare 0.015 N standard hydrochloric acid.

Procedure

Remove the adsorbed NH_4 on glass walls of the still. This removal can be accomplished for stainless steel stills by closing the cooling water, draining the condenser, and steaming the still out for at least 2 min (Saffigna and Waring 1977). In glass or stainless steel stills, NH_4 can be removed by steam-distilling for 1 min with 1 M formic acid and then distilling for 2 min with 95% ethanol (Mulvaney 1986). If the digest is known to have plenty of N, another approach is to bring the digest to volume and take two aliquots of the sample. Make the first aliquot alkaline by adding NaOH, and steam-distill it into boric acid. Collect 40 ml of distillate and titrate it for total NH_4, and then discard it. Make the second aliquot alkaline by adding NaOH, steam-distill it, and collect the distillate in 1 ml of 1 M HCl. Collection of the distillate in boric acid results in problems with the isotope-ratio analysis (Reeder 1984). If the digest is known to have a low N content, make the digest alkaline by adding NaOH, steam-distill the digest, collect the distillate in boric acid, and titrate the distillate either potentiometrically or with the mixed indicator to a pH of 5.1. After titration, immediately redistill the sample into 1 ml of 1 M HCl and concentrate the NH_4Cl salt to dryness. Cap and store the salt for isotopic analysis.

Analyses for NO_3, NO_2, and Exhangeable NH_4

Usually NO_3, NO_2, and exhangeable NH_4 are extracted from soil with a 1 M or 2 M KCl solution (Keeney and Bremner 1966, Keeney and Nelson 1982). However, in mineralization studies, Stanford and Smith (1972) extracted their incubating soils with weak $CaCl_2$ solutions. Such $CaCl_2$ solutions have been shown to contain considerable soluble organic N (Smith et al. 1980). This soluble N can have an effect on mineralization, and there is the possibility that some of the organic N may hydrolyze in basic solution and give erroneous NH_4 values. The NH_4 in a soil extract can be recovered using MgO as the alkalizing agent. Magnesium oxide is not as basic as NaOH and allows recovery of NH_4 without hydrolyzing hexosamines, amides, and amine N (Bremner and Keeney 1965). After NH_4 is recovered, the NO_3 and NO_2 in the extract can be reduced to NH_4 with Devarda's alloy and the NH_4 recovered by steam distillation (Keeney and Nelson 1982) or diffusion (O'Deen and Porter 1980, Brooks et al. 1987). We have found when using steam distillation flasks of various sizes that complete N recovery with Devarda's alloy is very difficult to achieve when the volume of the extract exceeds 250 ml.

The NH_4 and NO_3 content of soil extracts can be determined colorimetrically (Nelson 1983) or by titration after distillation (Keeney and Nelson 1982). Usually with soil extracts and water samples (irrigation water, irrigation runoff water, and ground water), small amounts of N (<100 μg N) are encountered. The small amounts of N in soil extracts and water samples can be readily analyzed with automated (Technicon and Lachat) colorimetric methods, but the small amounts of N in these samples are usually insufficient for isotope N analysis. In order to recover sufficient N for isotope analysis, the investigator must either evaporate large volumes of solution before distillation or diffusion or perform multiple distillation or diffusions and combine distillates. The automated conversion-MS system (McInteer and Montoya 1980, 1981, 1984) and the automated combustion system-interface-isotope MS (Preston and Owens 1983, Marshall and Whiteway 1985) permit the isotope analysis of samples containing 20 to 100 μg N—about one-tenth the amount of N required by the Rittenberg (1948) or Porter and O'Deen (1977) conversion systems.

Filtering soil extracts and water samples can result in possible error, since significant amounts of NH_4 or NO_3 have been found in many filter papers (Sparrow and Masiak 1987). Most of the NH_4 and NO_3 can be successfully removed by prewashing the filter paper with 2 M KCl.

The procedures for the recovery of NH_4, NO_3, and NO_2 from soil extracts and water samples are taken from Bremner and Keeney (1965, 1966).

Reagents

1. Potassium chloride 1 M: Dissolve 750 g of reagent grade KCl in 8 liters of deionized water. Dilute the solution to a final volume of 10 liters.
2. Magnesium oxide: Heat heavy magnesium oxide (U.S.P.) in an electric muffle furnace at 600 °C to 700 °C for 2 hr. Cool the product in a desiccator containing KOH pellets, and store the CO_2-free MgO in a tightly stoppered bottle.
3. Boric acid-indicator solution: Prepare as instructed in the distillation procedures found in the Kjeldahl section.
4. Devarda's alloy: Use a ball mill to grind reagent grade Devarda's alloy until the product passes a 100-mesh screen and at least 75% of it passes a 300-mesh screen.
5. Sulfamic acid: Dissolve 2 g of purified sulfamic acid in 100 ml of water, and store the solution in a refrigera-

tor. This reagent is only used to destroy NO_2.
6. Standard (NH_4 + NO_3)-N solution: Dissolve 0.236 g of dry $(NH_4)_2SO_3$ and 0.361 g of dry KNO_3 in deionized water, and dilute the solution to 1 liter in a volumetric flask. Thoroughly mix the solution. One ml of this solution contains 50 μg each of NH_4-N and NO_3-N. Store the solution in a refrigerator.
7. Standard (NH_4 + NO_3 + NO_2)-N solution: Dissolve 0.236 g of dry $(NH_4)_2SO_4$, 0.361 g of dry KNO_3, and 0.123 g of dry $NaNO_2$, and dilute the reagents to 1 liter with deionized water. Thoroughly mix the solution. One ml of solution contains 25 μg of NO_2-N and 50 μg each of NH_4-N and NO_3-N.
8. Prepare a 0.0075 N HCl standard.
9. Phenylmercuric acetate (PMA) solution: Dissolve 50 mg of phenylmercuric acetate in 1 liter of water.
10. Potassium chloride-Phenylmercuric acetate (1 M KCl-PMA) solution: Dissolve 750 g of reagent grade KCl in 9 liters of water, and add 1 liter of PMA solution.

Extraction Procedure

Place 10 g of soil in a 125-ml Erlenmeyer flask, and add 50 ml of the 1 M KCl. Stopper and shake the container on a mechanical shaker for one-half hour. Allow the soil suspension to settle until the supernatant is clear, and remove an aliquot for analysis. Filter the KCl-soil suspension on filter paper that has been prewashed with KCl. If solutions can't be analyzed immediately, store the filtrate in a refrigerator temporarily until the analyses can be performed. If the solutions need to be stored several weeks before analyses, add phenylmercuric acetate to the solution to inhibit microorganism growth (Douglas and Bremner 1970).

Analysis of Extracts

Remove any adsorbed NH_4 in the still by using the procedures previously described.

Analysis of NH_4-N: Add 5 ml of boric acid to a 100-ml beaker marked to a volume of 40 ml. Place the beaker under the condenser so that the end of the condenser tube is about 4 cm above the boric acid. Pipette an aliquot of the clear extract (supernatant or filtrate) into the distillation flask (flasks varying in volume from 100 to 500 ml may be used, but the volume of extract should never exceed 250 ml). Add 0.2 g of CO_2-free MgO to the solution in the flask, and immediately connect the flask to the still. Steam-distill until the distillate reaches the 40-ml mark on the beaker. Rinse off the end of the condenser tube. Determine the NH_4 content of the distillate by potentiometric or colorimetric titration. If titration is done potentiometrically, the distillate can be immediately evaporated to obtain dry NH_4Cl salt. If titration is by indicator, the extract should be removed from the still (keep for Devarda's reduction of NO_3) and the titrated distillate poured in a clean flask. Redistill the NH_4 in the distillate into HCl, and evaporate the distillate for isotope analysis.

Analysis of (NO_3 + NO_2)-N: Place 5 ml of boric acid in a 100-ml beaker, and place the beaker in the same manner as just described for NH_4 analysis. Since the previously distilled extract has been made alkaline with MgO, only add 0.2 g of the finely ground Devarda's alloy, and immediately attach the mixture to the steam-distillation apparatus. Steam-distill the mixture and determine the NO_3-N content of the distillate by titration as described in the NH_4 section above.

Analysis of (NH_4+ NO_3 + NO_2)-N: Add 0.2 g Devarda's alloy and 0.2 g CO_2-free MgO to the extract or filtrate. Steam-distill the mixture, and titrate the distillate to determine its N content.

Analysis of (NH_4 + NO_3)-N: Destroy any NO_2 in the sample by adding 1 ml of the sulfamic acid solution. Add Devarda's alloy and MgO as outlined above for the analysis of NH_4 + NO_3 + NO_2.

Analysis of NO_3-N: Add 1 ml of sulfamic acid to the sample and swirl the flask for a few seconds to destroy NO_2. Add MgO to the sample, and distill following the procedure described above to remove NH_4. Add Devarda's alloy to the mixture, and steam distill and trap the distillate in boric acid for titration. Redistill the distillate into HCl, and evaporate the NH_4Cl salt for isotope analysis.

Analysis of Blanks and Standards

Standards should be included in each set of digests. Also reagent blanks should be included to determine the amount of NH_4-N in reagents. If the samples to be analyzed contain very small amounts of NH_4-N or NO_3-N, the NH_4 in the reagents can cause considerable isotopic dilution of sample N. Since sulfamic acid may undergo decomposition during the analysis and may release NH_4 in the process, it is especially important that blanks be run to determine if sulfamic acid is contributing NH_4 to the analysis.

Often water samples and extracts of some soils contain levels of N that are too low for isotope-ratio analysis. Hauck (1982) describes various procedures to overcome this problem. He indicated that the most convenient way to prepare water, extracts, and even digests that are low in N content is to use the isotope dilution procedure of Hevesy (1932). Dilute an aliquot of the sample containing an unknown amount of inorganic N of unknown N isotopic composition with an internal

standard, i.e., one with a known amount of NH_4-N or NO_3-N having a known isotopic ^{15}N composition (up to 5 times the amount of N in the sample may be added). Distill the mixture using appropriate distillation procedures, and determine the total N and the ^{15}N of the distillate. Use the following equation to calculate the ^{15}N concentration of the unknown sample:

$$A_o = [A_m(x + y) - A_i \times y]/x$$

where A_o, A_i, and A_m = atom % ^{15}N in sample, internal standard, and mixture of sample and standard, respectively, and x and y = mg or μg of N in sample and internal standard, respectively. The ^{15}N abundance and N content of the internal standard must be accurately known.

Oxidation of NH_4 to N_2 for Isotope-Ratio Analysis

The oxidation of NH_4 by LiOBr is described by the following chemical equation:

$$2\,NH_4Cl + 3\,LiOBr + 2\,LiOH \rightarrow N_2 + 3\,LiBr + 2\,LiCl + 5\,H_2O$$

Most of the information concerning this reaction was obtained using the reagent NaOBr, which is not as stable as LiOBr (Ross and Martin 1970). Hauck (1982) indicates that about 1.5 to 3.0% of the gas produced using the NaOBr reagent is N_2O. Also some O_2 is formed by the decomposition of the NaOBr, and Bremner (1965) discussed how KI can be used to prevent the formation of O_2. Ross and Martin (1970) indicated that LiOBr's stability prevents the formation of O_2. Any excess acid in the sample will react with the hypobromite to liberate Br_2, which is corrosive to any metal inlet parts of the MS. To avoid corrosion of metal inlet parts, use HCl as the trapping agent for NH_3.

The oxidation reaction has traditionally been carried out in an evacuated Rittenberg Y-tube (Sprinson and Rittenberg 1948). This procedure is quite tedious and time consuming, since it requires transferring the sample and the LiOBr to the two legs of the Y-tube, lubricating the vacuum stopcock, freezing the sample to prevent water vapor from getting into the vacuum system, evacuating the container, thawing the contents in order to degas the sample and the LiOBr, and refreezing, reevacuating, and thawing the sample so that the oxidation reaction can be carried out. After the N_2 is emitted to the MS, the Y-tube needs to be degreased and cleaned. Ross and Martin (1970) developed a conversion apparatus that completely eliminated the need for the classical Rittenberg Y-tube. Utilizing the Ross and Martin principle of dropping degassed LiOBr onto dry NH_4, Porter and O'Deen (1977) designed and built a simplified conversion system with Cajon Ultra-Torr stainless steel vacuum fittings (shown in fig. 1). Connections between the glass LiOBr reservoir and shell sample vials containing the NH_4Cl salts are made via Cajon Ultra-Torr high vacuum Viton-O-ring fittings. The stopcocks in the LiOBr reservoir are Teflon with Viton O-rings for vacuum seals. With this design the vacuum seals between the glass reservoir and the glass sample vial are improved. The design also permits LiOBr to be degassed simply and maintained under an atmosphere of He. Inexpensive disposable shell vials are used in order to avoid cleanup. To eliminate the need for evacuating the conversion vial through the high-vacuum inlet pumping system of the isotope-ratio MS, Porter and O'Deen (1977) installed an auxiliary forepump-oil diffusion pump high-vacuum system below the conversion system. The auxiliary vacuum system is used to evacuate the conversion system and vacuum line leading to the MS while an isotope-ratio analysis is being performed on an N_2 sample in the MS inlet. A dry-ice-isopropanol bath is used to freeze water vapor in the sample vial after the conversion. A liquid N_2 trap is used between the conversion system and MS to freeze out any N_2O or NO_x compounds. This trap must be heated between each sample because a small amount of sample N_2 may freeze out and must be removed before the next sample is introduced.

Reagents

1. LiOBr: To 60 ml of cold (0-5 °C) 10% w/v solution of analytical reagent grade lithium hydroxide and water, add 2 ml of analytical reagent grade bromine; shake the mixture until the bromine is thoroughly mixed.
2. Isopropanol-dry-ice slush: Grind or crush the dry ice so that it will pass through a screen with a 4.7-mm opening. Place the dry ice in a Dewar container, and slowly pour isopropanol into the ground dry ice to produce a slush.
3. Liquid N_2.

Procedure

Fill the LiOBr reservoir by evacuating the reservoir and drawing the 60 ml of LiOBr through the bottom stopcock. Degass the LiOBr in the reservoir by applying a vacuum and then emitting an atmosphere of helium into the reservoir. Shake the LiOBr and reevacuate. Repeat this process several times. Finally, bring the reservoir up to atmospheric pressure with He and seal. Buresh et al. (1982) modified the reservoir so that the LiOBr could be degassed by flowing a stream of He at a pressure of approximately 0.2 kg cm^{-2} through a glass frit and out the top stopcock for 1 hr.

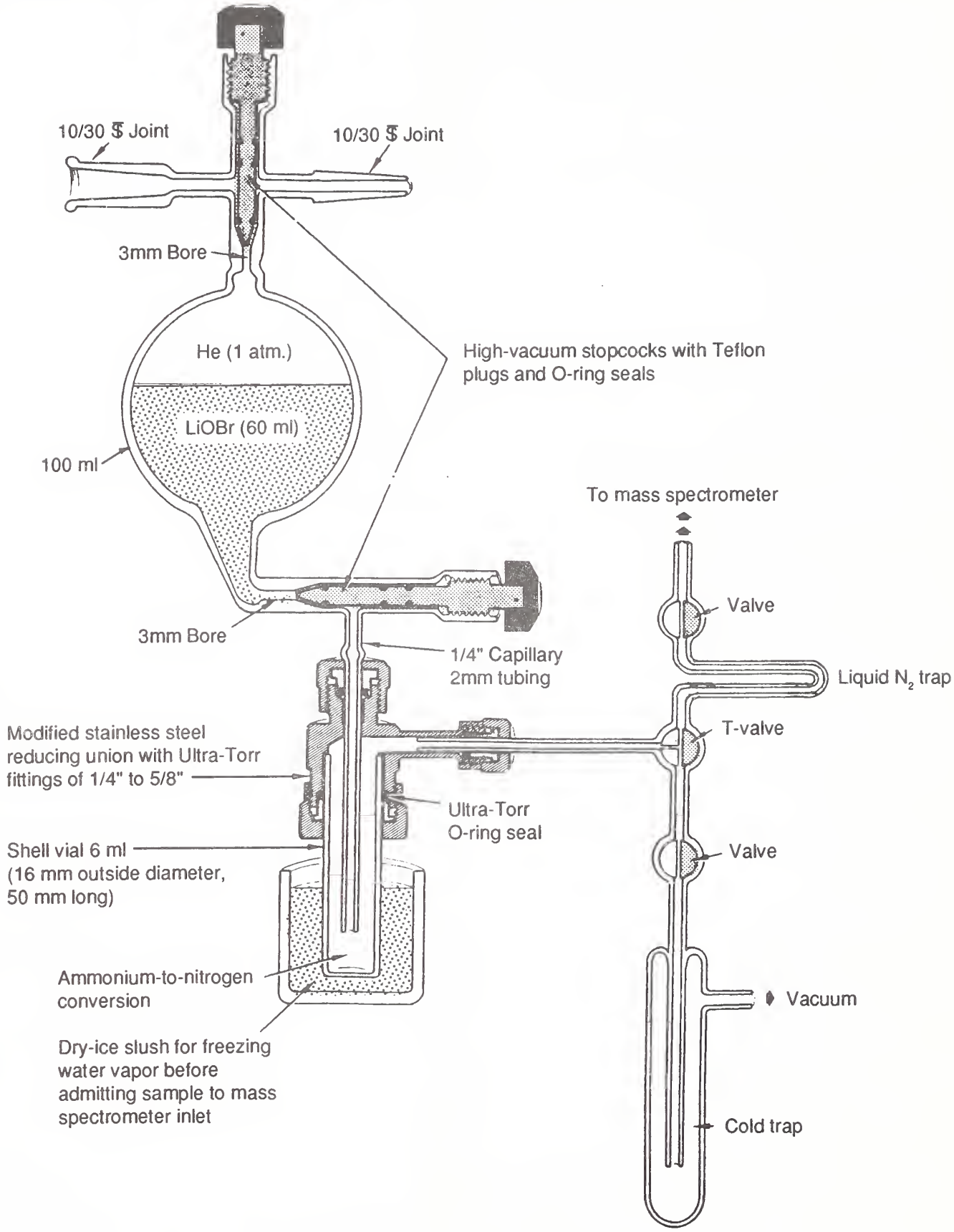

Figure 1. Ammonium-to-N_2 conversion apparatus.

Attach the sample vial to the conversion apparatus, and evacuate the sample by using the auxiliary vacuum system. Isolate the evacuated sample with a vacuum stopcock. Immerse the sample vial (usually containing 1-3 mg N) in the dry-ice-isopropanol slush, and allow 0.5-1.0 ml of the degassed LiOBr to slowly drop on the dry NH_4 salt. After the reaction warm the sample until it is liquid, and then refreeze the sample with the dry-ice bath. Allow the N_2 sample to pass into the MS inlet system via the liquid N_2 trap. Reevacuate the empty vial to remove LiOBr from the glass LiOBr delivery stem. Remove and discard the sample vial, and wipe the LiOBr glass delivery stem with acetone. Remove the liquid N_2 and warm the trap. Attach the next sample and evacuate the conversion system using the auxiliary vacuum pump system while the previous N_2 sample is being analyzed in the MS.

Isotope-Ratio Analysis

The operation of an isotope-ratio MS and the MS inlet system is not described here. Fiedler (1984) describes viscous and molecular leak inlet systems and describes how one can calculate the required pressure and the amount of sample N required to obtain that pressure. He also describes a dual-inlet system, which allows switching between sample and standard gas. Comparisons between the standard and sample allow very precise ^{15}N enrichment measurements, which are particularly useful when making natural abundance or depleted ^{15}N determinations. Descriptions and principles of operating MS's can be found in instrument manuals and published reviews (Beynon 1960, Bremner 1965, Fiedler and Proksch 1975, Fiedler 1984).

Once the N_2 sample is in the MS, the N_2 is ionized and then separated by acceleration through a magnetic field. Monoenergetic ions, as they pass through a magnetic field, separate into different radii depending on their mass/charge ratio. Ions of different mass can be collected by correctly positioning the Faraday ion collectors. Dual and triple collector MS's can be used to collect ions of different masses simultaneously with appropriately positioned ion collectors. As the charged molecules are collected on the Faraday collectors, a current is generated that is proportional to the number of ionized molecules collected. The ion current is amplified so that it can easily be monitored. Nitrogen atoms are paired to form dinitrogen molecules of $^{14}N^{14}N$ (mass 28), $^{15}N^{14}N$ (mass 29), and $^{15}N^{15}N$ (mass 30). Once the proportion of various molecular species is determined, the percentage abundance of ^{15}N is calculated from the equation:

$$\text{atom \% } ^{15}N = (^{29}N_2 + 2 \times {}^{30}N_2)100/[2(^{28}N_2 + {}^{29}N_2 + {}^{30}N_2)]$$

The atom % ^{15}N represents the ratio of the ^{15}N atoms to the total number of N atoms. Hauck and Bremner (1976) indicated that for abundances above 5 atom % ^{15}N, the highest accuracy is achieved by measuring all N_2 species ($^{28}N_2$, $^{29}N_2$, and $^{30}N_2$). When the ^{15}N abundance of the sample is below 5%, it is not necessary to measure the ion currents of all dinitrogen species. This is because the N_2 that is produced by the LiOBr reaction comes to an equilibrium between the various N_2 species as represented by the following chemical equation:

$$^{28}N_2 + {}^{30}N_2 \leftrightarrow 2\,{}^{29}N_2$$

The theoretical and experimental equilibrium constant of this reaction is 4.00. Bremner (1965), using this constant, shows the derivation of the following equation (where only the ion currents for mass 28 and mass 29 are required in the calculation of the ^{15}N abundance):

$$\text{atom \% } ^{15}N = 100/(2R + 1)$$

where R is the ratio of the ion currents for mass 28/mass 29, corrected for residual mass 28 and mass 29 background in the MS and any atmospheric N_2 leakage into the sample (determined by the magnitude of the Argon, mass 40, in the MS) (Bremner 1965, Hauck 1982).

The isotope ratio MS may be equipped with dual inlets so that the sample N_2 can be rapidly compared to a standard or reference N_2 (usually tank N_2). The isotopic abundance of the standard gas must be in the same range as that of the sample. This comparison permits one to correct for any errors caused by changes in the electronic or vacuum system, but it does not correct for any air leakage into the sample. Air contamination can be determined by changes in the O_2 peak (mass 32) or Ar peak (mass 40) (Bremner 1965). The percentage enrichment (E) of the sample is defined by the formula:

$$E = 100(R_{sample} - R_{std.}) / R_{std.}$$

where R_{sample} and $R_{std.}$ are the measured ratios of $^{28}N/^{29}N$ for the sample N_2 and the standard N_2. Thus E is the relative enrichment of the sample N_2 with respect to the standard N_2 and is not an absolute value. Rearranging the equation we obtain:

$$R_{sample} = (1 + E/100)\, R_{std.}$$

If the absolute isotopic abundance for the standard N_2 is substituted into this equation, the isotopic abundance value for the sample N_2 can be calculated.

Calculation of Results

Hauck and Bremner (1976) and Rennie and Paul (1971) have provided excellent instructions for calculating experimental results from ^{15}N abundance measurements. In plant recovery experiments, Rennie and Paul indicated that the percentage plant N derived from fertilizer (% Ndff) can be calculated using the following equation:

$$\% \text{ Ndff} = 100 \text{ (atom \% } ^{15}\text{N excess in sample/atom \% } ^{15}\text{N excess in fert.)}$$

where atom % ^{15}N excess refers to the actual atom % ^{15}N minus the natural abundance atom % ^{15}N of untreated (check) sample.

The Ndff percentage values are independent of plant yield. They must be used with caution, since they reflect the dilution of the applied ^{15}N, and the dilution is a function of soil N mineralization, fertilizer placement, soil nitrogen-fertilizer interactions ("priming"), etc. The percentage of N derived from soil (% Ndfs) can be calculated by using the equation:

$$\% \text{ Ndfs} = 100 - \% \text{ Ndff}$$

The percentage of fertilizer N recovered or utilized can be calculated by using the equation:

$$\% \text{ fert. N recovered} = (\% \text{ Ndff} \times \text{plant N yield})/\text{fert. N applied}$$

where plant N yield and fertilizer N applied are expressed in the same units. A more accurate version of the calculation is the expression from Hauck and Bremner (1976) in which % fert. N recovered is calculated using the following equation:

$$\% \text{ fert. N recovered} = 100 \times P(c - b)/[f(a - b)]$$

where P is total milliequivalents of N found in the plant material, f is milliequivalents of N applied, c is atom % ^{15}N in the plants grown on soil fertilized with tagged N, a is atom % ^{15}N of the fertilizer N, and b is atom % ^{15}N of plants grown on a nonfertilized plot.

When utilizing ^{15}N-depleted fertilizer, use the following equation for calculating % fert. N recovered:

$$\% \text{ fert. N recovered} = 100 \times P(b - c)/[f(b - a)]$$

The atom % ^{15}N can be corrected for the dilution caused by Kjeldahl reagent N (blank) by using the following equation of Huser (1966):

$$A = x(g - n)/(a - g)$$

where A is the quantity of Kjeldahl reagent N added to the sample, x is the quantity of N in the sample before the Kjeldahl reagents were added, a is the atom % ^{15}N of the N in the Kjeldahl reagents, n is the atom % ^{15}N of the sample N before the addition (A), and g is the atom % ^{15}N obtained by analysis of the diluted sample.

Rearranging the equation, one obtains n, the atom % ^{15}N before the addition of reagent N:

$$n = (Ag + xg - Aa)/x$$

The following examples will illustrate the effect of the reagent N on the atom % ^{15}N of the sample N as the total N of the samples varies (the example assumes g = 4.5 atom % ^{15}N, A = 10 μg N, and a = 0.37 atom % ^{15}N). If x = 50 μg N, we can calculate with the equation that n = 5.326 atom % ^{15}N; if x = 1,000 μg N, however, n = 4.541 atom % ^{15}N.

The amount of N in the sample can be determined with the highest accuracy only if the amount of N in the fertilizer, soil, and plant samples is corrected to reflect the isotopic composition or average atom weight of N in the samples (Hauck and Bremner 1976). The average atom weight of N (M_N) in the samples can be determined by using the following equation:

$$M_N = 0.01[(\text{atom \% } ^{15}\text{N} \times 15.000) + (\text{atom \% } ^{14}\text{N} \times 14.003)]$$

As the following formula shows, the ^{15}N balance for a greenhouse or field study can be calculated by summing the amount of ^{15}N in the various components of the system:

$$\% \ ^{15}\text{N recovered} = 100 \text{ (wt. } ^{15}\text{N in plant parts, straw + chaff + grain + roots, + wt. } ^{15}\text{N in soil + wt. } ^{15}\text{N in leachates and runoff water + wt. gaseous } ^{15}\text{N products)/wt. } ^{15}\text{N applied.}$$

Automated Analysis for ^{15}N

Most of the techniques discussed thus far for the measurement of total, inorganic, and organic N are based on Kjeldahl digestion, the recovery of NH_4, and the conversion of NH_4 to N_2 via the classical Rittenberg oxidation method using alkaline hypobromite. These techniques are complicated and tedious and require a disproportionately large share of an investigator's time. Barsdate and Dugdale (1965) were probably the first to investigate the Dumas method for obtaining N_2 from samples for isotopic analysis. They tried coupling the semiautomatic Coleman Nitrogen Analyzer to the MS through a liquid N_2 trap that removed CO_2. A

Toepler pump was used to regulate the gas pressure delivered to the MS. The method had problems—the CO_2 sweep gas had a high N_2 background, and Toepler pump manipulations were time consuming. With this system, two operators were able to run five samples per hour. Desalty et al. (1969) further modified the technique by Barsdate and Dugdale and identified problems that needed to be rectified and precautions that should be observed to obtain accurate results. One of the main problems was caused by compounds such as NH_4NO_3, glutamine, and p-aminophenylalanine. These compounds, when enriched in only one of their two carbon atoms and combusted, do not give random pairing for the N_2 produced. Proksch (1969) investigated combustion of plant materials and concluded that nonrandom pairing is not a problem when the enrichment is low, i.e, < 5 atom % excess ^{15}N. The nonrandom pairing is a problem only when the ratio of $^{28}N_2$ and $^{29}N_2$ are used to calculate ^{15}N abundance. Nonrandom pairing is not a problem if $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ are being measured and used to calculate the ^{15}N abundance. Fiedler and Proksch (1972, 1975) developed a Dumas procedure in which inorganic, organic, and plant samples were transferred to Pyrex or Supremax glass tubes with combustion reagents of copper oxide, platinum catalyst on alumina, calcium oxide, lead dioxide, and potassium perchlorate. The sample, reagents, and sample tubes were degassed under vacuum and sealed. Combustion products, water and CO_2, reacted with the reagents, leaving pure N_2 for analysis. Fieldler and Proksch (1972) constructed a rotary multisource holder with chambers of known volume. The chambers were arranged so that they could be evacuated. The sealed sample tube was crushed in the evacuated chambers, and the pressure of the N_2 gas was measured by a manometer to obtain a total N value. The sample was then introduced into an MS for isotope analysis. The rotary apparatus allowed them to process 80 samples per day. However, the components of the apparatus were not commercially available or simply machined.

In the early eighties Carlo Erba Strumentazione (of Milan, Italy) began commercially producing the automated Carlo Erba NA 1300 and 1500 nitrogen analyzers (ANA's). In an ANA, the combustion gases N_2 and CO_2 are swept by He into a gas chromatograph (GC) and measured. Several investigators (Preston and Owens 1983, Barrie and Workman 1984, Marshall and Whiteway 1985) began interfacing the ANA with isotope-ratio MS's and introduced computer control of the ANA, MS, and interface. Kirsten (1983) described the operation of the ANA and some of the precautions for operating it. The sample (plant tissue, soil, or inorganic or organic compounds) is weighed into a tin capsule, and the encapsulated samples are loaded into an autosampler. During the operation of the autosampler, a capsule is dropped from the autosampler into the combustion tube containing Cr_2O_3 and silver wool at about 1,000 °C. A pulse of oxygen is then injected into the combustion tube, and a flash combustion of the sample and tin capsule occurs, raising the temperature in the combustion tube to about 1,500-1,700 °C. Combustion products are swept by He through the silver wool, where halogens and SO_2 are removed, and then through a reduction tube (metallic Cu, 700 °C), where any excess O_2 is converted to CuO and oxides of nitrogen are reduced to N_2. The N_2 and CO_2 are further swept through magnesium perchlorate to remove H_2O and then through a GC column, where CO_2 and N_2 are separated. The separated CO_2 and N_2 are measured by a thermal conductivity detector to give total N and total C. Carbon dioxide is removed from the He effluents by Carbosorb or Ascarite, and a small portion (approximately 1%) of the He/N_2 effluent is admitted through a 0.15-mm-I.D. capillary tube to the isotope-ratio MS.

Exhaustion of the Cu in the reduction column varies with the type and kind of samples as well as the size of the O_2 pulse. When the column is exhausted, NO is no longer reduced to N_2 and is swept through the various scrubbers and GC column to the MS, where the NO interferes in the measurement of mass 30. However, the NO peak is out of sync with the peak of $^{30}N_2$, and therefore it is easy to determine whether the metallic Cu needs regeneration. The metallic Cu can be regenerated by disconnecting the water trap and the combustion column and passing H_2 through the hot Cu.

Barrie and Lemley (1989) discussed data collection, calibration against a reference sample, and blank corrections to account for the small amount of N_2 impurity from tin capsules and the O_2 pulse. The reference samples should be selected so that they have about the same N composition and isotopic abundance as the unknown sample. The amount of sample required depends upon its N content; our experience indicates that good isotopic ratios can be obtained if the sample contains from 20 to 200 μg N. The samples must be finely ground to about 200 mesh to be homogeneous. Accuracy of weighing is critical for the total N analysis but not for the isotopic-ratio analysis. The weighing of 4-5 mg of grain, 5-15 mg straw or plant materials, or 30-40 mg soil can be a tedious process.

Commercial automated N/C analyzers coupled to isotope-ratio MS's are being produced by Europa Scientific Ltd., Crewe, England; VG Isogas Middlewich, Cheshire, England; and Finnigan MAT, San Jose, California; and are capable of analyzing the total N and N^{15} content of a sample every 5 minutes.

Direct Measurement of Denitrification Using ^{15}N

The technique of directly measuring denitrification in the field was first proposed by Hauck et al. (1958). Hauck and Bouldin (1961) described the method further, but it was not until two decades later that the applicability of the method became evident. Siegel et al. (1982) revitalized the technique and modified the associated calculations. Mulvaney (1984) adapted the calculations to a triple collector MS, and Mulvaney and Boast (1986) refined the calculation equations to permit the use of N salts lower in ^{15}N abundance. Mulvaney and Kurtz (1982) showed that N_2O evolution from soil could be measured by this technique. Mosier et al. (1986 a and b) used the technique to estimate N loss from maize and barley fields in Colorado. The "Hauck" method involves applying fertilizer that is highly enriched in ^{15}N (>20 atom % ^{15}N) and covering the ^{15}N-fertilized soil with a chamber to isolate the atmosphere above the soil for a designated time, permitting determination of the rate of change of ^{15}N atoms (N gases) in the isolated atmosphere over time. The calculations utilize the fact that N gases (principally $^{30}N_2$) emitted from the ^{15}N-enriched soil into the chamber do not mix isotopically with the atmospheric N_2 (normal ^{15}N abundance) in the chamber. Using calculations of nonrandom ^{15}N distribution permits estimates of denitrification, i.e., the amount of N gas evolved from the added ^{15}N- enriched fertilizer and N from soil constituents. The NO_3 in the soil serves as the source of the emitted N gas. The ^{15}N mole fraction is calculated directly from N_2 mass spectral data (29/28 and 30/28+29 ratios). The ^{15}N content of the soil NO_3 must be known in order to calculate the total amount of N_2 evolved from the site at any given time, but since the emitted gas directly reflects the composition of the soil NO_3, it is not necessary to disturb the soil in the plot during the course of the experiment. The technique is reasonably sensitive, as 5 g N/d/ha can be detected when the ^{15}N content of the soil NO_3 is > 20 atom %.

Gas Collection and Analysis

Because analytical methods are not sufficiently sensitive to directly measure the relatively small instantaneous upward flux of N_2 from the soil or floodwater surface, a soil-cover technique must be used. Chambers enclose a distinct volume of air above a known area of soil and prevent the emanating N_2O and N_2 from mixing with the outside atmosphere. When $^{30}N_2$ and $^{29}N_2$, or $^{30}N_2O$ and $^{29}N_2O$ are released from the soil, their concentration in the chamber will increase. The changes in concentration of these N gas species over time is used to calculate the flux out of the soil. We typically use a closed-cover technique described by Hutchinson and Mosier (1981). In this technique the cover is vented to the atmosphere so that the enclosed soil is subjected to the same atmospheric pressure fluctuations as uncovered soil. In a denitrification experiment at Cuttack, India, we used rectangular chambers, 14 cm wide by 40 cm long by 12 cm high. These covers were made from plates of glass that were glued together; the joints of the plates were sealed with silicone caulking. Two holes were made in the top of each chamber to accommodate rubber septa through which a needle could be inserted to withdraw a gas sample. The open end of each chamber was typically pushed into the soil so that a 9-cm gas headspace existed above the floodwater. During installation of the chambers the serum stoppers were left out to prevent a pressure buildup in the chambers.

When using the soil covers, withdraw (zero-time) gas samples immediately after installation by using a polypropylene syringe fitted with a two-way, gas-tight, plastic stopcock. When possible, take the samples to the laboratory and analyze them within a few hours of collection. If immediate analysis is not possible, store gas samples in rubber-stoppered, evacuated glass tubes (a commercial example is Vacutainers). When preparing samples for storage, inject 12 ml of gas into an 11-ml tube to produce a positive pressure that will limit leakage of outside air into the vial. Reseal the stopper injection hole with silicone caulking, as an occasional leak occurs unless the caulking is used.

A potential problem exists when Vacutainers are used as gas storage devices because the tubes are not completely evacuated and thus contain a significant amount of gas before the sample is added. The pressure in the tubes is quite constant, however. By measuring existing pressure in a large set of evacuated Vacutainers before and after injecting 12 ml of air, it was apparent that the injected samples constituted only 77.63 % of the contents of the tubes. This initial dilution does not affect the $^{15}X_N$ calculations but does reduce the calculated d value (see table 1 for definition of terms). The d value is consistently underestimated by a factor of 1.2882. A second potential problem with the Vacutainers is that the N_2O content of the tubes is above that of normal air. When the tubes are filled with 12 ml of normal atmospheric air, the tube air contains about 800 ppb v/v N_2O rather than the 310 ppb normally found in air. The excess N_2O is not a problem when measuring total N gas on the MS but must be corrected for when quantifying N_2O emissions from the soil. The excess N_2O can be readily corrected for by using the time-zero gas samples and analyzing their N_2O content.

Table 1. MS equations used to calculate N gas emissions

1. $\Delta r = (^{29}N_2/^{28}N_2)_{sample} - (^{29}N_2/^{28}N_2)_{reference}$

2. $\Delta r' = [^{30}N_2/(^{28}N_2 + ^{29}N_2)]_{sample} - [^{30}N_2/(^{28}N_2 + ^{29}N_2)_{reference}$

   where sample is air sample from collection chamber at some time (t) after installing chamber; reference is air sample taken from chamber immediately after installation; and $^{29}N_2/^{28}N_2$ and $^{30}N_2/(^{28}N_2 + ^{29}N_2)$ are MS ion current ratios (r).

3. $^{15}X_N$ = mole fraction of ^{15}N in the soil NO_3 pool
   $= 2.015(\Delta r'/\Delta r)/[1 + 2.015(\Delta r'/\Delta r)]$

4. d = the fraction of total N gas in the gas collection chamber attributable to denitrification
   $= \Delta r'/(^{15}X_N)^2$

5. Total N gas evolved from the soil into the collection chamber
   = total N_2 in the chamber volume × d.

6. N_2 Flux $= \Delta C/A \times \Delta t$

   where A = soil surface area covered by chamber, t = time that the chamber covered the soil, and ΔC = the change in concentration of $^{30}N_2$ and $^{29}N_2$ in the chamber during time (t).

Before analyzing the gas sample on the MS, it is necessary to construct an inlet system on the MS so that O_2, CO_2, and water vapor can be removed from the gas sample. The inlet should consist of a series of components: a 2- to 5-ml sample loop, an O_2 scrubber, and a cold trap, each connected by a gas-tight valve to permit isolating each portion of the inlet. Arrange the inlet so that it will permit a gas sample to be injected directly into the sample loop. Using a syringe fitted with a 2-way Luer-fitted stopcock, inject 1 or 2 ml of the sample into the sample loop. Immerse the loop in a cryogen (liquid N_2 or a dry-ice-cooled solution) to remove water vapor, CO_2, or N_2O, depending upon the desired analysis. Do not use a cryogenic trap for the analysis of total N gas evolution from the soil. After the gas is injected into the sample loop, the gas will pass into the O_2 scrubber. The scrubber is made by packing a tube (size is not critical) with about 10 g of O_2 trapping material obtained from L.C. Company (619 Estes Ave., Schaumburg, IL 60193). The scrubber can be operated at room temperature but is much more efficient at an elevated temperature. At 250 °C-300 °C, the trap effectively removes O_2 and reduces N_2O and NO to N_2. After several hundred samples are analyzed, the trap should be regenerated by passing H_2 gas through the hot trap (300 °C) for about 2 hours (Siegel et al. 1982). About 30 sec after the sample is introduced into the O_2 scrubber, the sample is passed through a high-efficiency liquid N_2 trap to remove CO_2 and H_2O. The purified sample N_2 is then passed into the MS inlet. Both the sample and reference gases are introduced into the MS through the gas inlet system so that they can be compared directly. If a dual-inlet MS is available, a time-zero gas sample can be introduced into the reference side of the MS inlet and the sample gas into the other side of the inlet. If a single-inlet instrument is used, samples and reference gases must be run sequentially.

Calculations for Determining N Gas Emissions

The basic equations (table 1) used to calculate the total N gas flux are from Siegel et al. (1982), Mulvaney (1984), and Mulvaney and Boast (1986). The total N_2 + N_2O + NO is not just the N_2 derived from the added fertilizer but is also from indigenous sources of N. The equations use a number of assumptions. The first assumption is that the total amount of $^{28}N_2$ inside the gas collection chamber does not change during the sample collection period. Under most situations the N_2 content of the chamber atmosphere is at least 1,000 times greater than the amount of N_2 evolved during a 24-hr period. The second major assumption is that the ^{15}N label of the NO_3 in the soil is uniform. We assume that when the ^{15}N-labeled fertilizer is added to the soil it mixes uniformly with the unlabeled NO_3 already present in the soil. We also assume that nitrate formed from organic N mineralization during the course of the study will uniformly mix with the tagged NO_3. The assumption of uniform ^{15}N label of the soil NO_3 pool may be debatable, however. Some studies (Mosier unpublished, Mosier et al. 1986b) have indicated that as long as some ^{15}N label mixes with the nonlabeled NO_3 in the soil NO_3 pool, uniform distribution of the tagged N in the NO_3 pool is not necessary to obtain accurate emission estimates.

Data from gas samples collected from a 1988 denitrification experiment in Cuttack, India (Central Rice Research Institute, CRRI), and analyzed on the New Delhi MS (at the Indian Agricultural Research Institute, IARI) are presented here for use in example calculations. The MS was fitted with a gas sampling inlet system. The samples from CRRI were collected 0 and 16 hr after placing collection chambers inside rice plots that had been fertilized with 85 atom % ^{15}N urea. The gas samples were injected into Vacutainers and taken to IARI. The MS data and calculations follow:

	Sample Gas 16 hr Plot 1		Reference Gas 0 hr Plot 1	
	Isotope ratios			
	29/28	*30/28+29*	*29/28*	*30/28+29*
Rep 1	0.00741	0.0000426	0.00740	0.0000267
Rep 2	0.00741	0.0000370	0.00740	0.0000262
Rep 3	0.00741	0.0000407	0.00740	0.0000262
Mean	0.00741	0.0000401	0.00740	0.0000264

$\Delta r = 0.00741 - 0.00740 = 0.00001$
$\Delta r' = 0.0000401 - 0.0000264 = 0.0000137$
$^{15}X_N = 0.741$
$d = 0.0000248$

The assumptions were made that the amount of N_2 inside the 5,040-ml chamber at time-zero was 78 % N_2, that the temperature inside the chamber was 25 °C, and that the pressure was 760 mmHg. The volume inside the chamber was corrected to standard temperature and pressure (STP, 273 K and 760 mmHg). We calculate that there was a total of 3,601 ml of N_2 inside the chamber. Since one mole of N_2 at STP occupies 22,400 ml, then 3,601 ml of N_2 gas is 0.161 moles N_2 or 4.502 g of N_2. The amount of N gas evolved from the soil into the gas chamber was:

N_2 evolved = g N_2 in chamber × d × correction factor for Vacutainer storage
= 4.502 g × 0.0000248 × 1.2882
= 143.81 μg N/plot/16 hr

The N gas evolved at the following rate:

N_2 Flux = 143.81 μg/(560 cm^2 × 16 hr) = 0.0161 μg N/cm^2/hr

On a hectare basis then, 1.61 g N/hr evolved from the soil during this 16-hr sampling period, which began 52 hr after urea fertilization.

Experimental Precautions in Measuring Denitrification

A few precautions must be taken when conducting ^{15}N studies to measure denitrification. The calculations shown in table 1 can be used to obtain accurate results if the ^{15}N content of the soil NO_3 is above 20 atom % ^{15}N. If lower ^{15}N enrichments are encountered or if the MS has an adequate computer control and computation system, the more complex but more accurate equations presented by Mulvaney and Boast (1986) should be used. It is imperative that the O_2 and CO_2 be removed from the gas samples. The O_2 reduction tube and liquid N trap effectively and simply serve this purpose. The VG-622 "Jump Program" (used in New Delhi) can be used to control the MS accelerator voltage and to permit direct measurement of O_2 in each sample so that the efficiency of the O_2 trap is readily monitored. The CO_2 and H_2O trap will work efficiently if the trap is removed from the liquid N_2 and heated with a hot-air gun (or a hair dryer) after each sample. Reference samples should be run frequently (about every 10 samples) to monitor instrument drift when conducting analyses throughout the day.

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